An approach to gut microbiota profile in children with autism spectrum disorder

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1758-2229.12810

Abstract

In recent years, there has been an increase in studies on the implications of gut microbiota (GM) on the behavior of children with autism spectrum disorders (ASD) due to a dysbiosis in GM that can trigger onset, development or progression of ASD through the microbiota-gut-brain axis. The aim of this study is to carry out a systematic review of articles from the last 6 years that analyze GM in children with ASD compared to GM in control groups. Children with ASD showed higher abundance of *Roseburia* and *Candida* genera, and lower abundance of *Dialister*, *Bilophila*, *Veillonella*, *Streptococcus*, *Coprococcus* and *Prevotella* genera. Those differences can be attributed to factors such as different nationalities, nature of control groups, place where the sample was taken, gastro-intestinal (GI) problems or bacterial detection methods. It is still too early to define a specific GM profile of children with ASD, and future studies should focus on homogenizing the characteristics of samples and control groups. Further, new multicenter studies should also focus on the impact of GM on: GI physiology, neurophysiology and behavior of children with ASD, and on performing psychometric analyses of the correlation between the severity of ASD behavioral symptoms and GM profiles.

Keywords: Gut Microbiota; Children; Autism Spectrum Disorders (ASD); Dysbiosis; Microbiota-gut-brain axis; Systematic review

Introduction

Gut microbiota (GM) represents about 99% of the entire human microbiome (Frye et al., 2017). It weighs approximately 1 kg (containing about 9.9 million bacterial genes) (Li et al., 2017), and it is composed of: 92.9% bacteria, 0.5% eukaryotes, 0.8% archaeas and 5.8% viruses (Arumugam et al., 2011). Gut microbiota abundance ranges from about 10^8 bacteria/g measured in ileum to ~10^{11} bacteria/g measured in stool (Sender et al., 2016), with Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, and Fusobacteria being the major bacterial phyla in the mammalian gastro-intestinal (GI) tract (Hill et al., 2010). Recently, the development of human GM at the phylogenetic level has been related to the type of food intake and the preparation methods (e.g. food cooked on fire or boiled) (Danchin, 2018). However, there are cultural and geographical differences in GM composition. *Prevotella*, *Proteobacteria*, *Spirochaetes*, *Clostridiales* and *Ruminobacter*, were highly abundant in the gut microbiota of the hunter-gatherer populations, while those of urban communities are often enriched in *Bacteroides*, *Bifidobacterium*, and Firmicutes. There are also variations in the composition of GM between different nationalities and latitudes (Lindström and Langenheder, 2012; Suzuki and Worobey, 2014; Gupta et al., 2017; Cerdo et al., 2018).

GM may play an important role in the production of several metabolites such as propionic acid (PPA), other short chain fatty acids (SCFAs), and gaseous molecules, which can induce effects on the GI tract, brain and behavior (MacFabe, 2015). These metabolites can produce effects via biochemical, immunological, and neuroendocrine mechanisms that involve endogenous and microbial modulators and transmitters (Frye et al., 2017). Changes in redox signaling, epigenetic transcriptional factors, lipid and mitochondrial metabolism, ion channel/gap junction/transporter regulation protein, and post-translational modification have also been reported (Frye et al., 2016; Rose et al., 2017). In addition, other biomarkers such as serotonin, p-cresol and cortisol have also been related to etiology and behavior in mental disorders such as Autism Spectrum Disorder (ASD) (Yang et al., 2015; Ferguson et al., 2016; Ooi et al., 2017; Kang et al., 2018).

Recent studies have indicated that it is necessary to continue investigating the interaction between GM and the epithelial cells of the GI tract because GM seems to act as an epigenetic regulator of several diseases (Kumar et al., 2014). Thus, dysbiosis or imbalances in the GM can induce intestinal inflammation that is associated with the pathogenesis of several diseases such as obesity, Crohn's disease, irritable bowel syndrome, Obsessive-Compulsive Disorder, Depression, Anxiety, type 2 diabetes mellitus and neurodegenerative diseases including Alzheimer's and ASD (Durbán et al., 2012; Mangiola et al., 2016; Schäffler et al., 2016; Ding et al., 2017; Pradhan et al., 2017; Sanchez-Samper et al., 2017).

GM maturation occurs during the first years of life, coinciding with the critical window of early brain development and, at the third year of life, the GM of children is similar to that of adults (Diaz Heijtz, 2016). It is also known that the microbiota may continue to

develop into adolescence (Hollister et al., 2015). In this sense, the first years of life are an important period for the appearance of neurodevelopmental disorders (Borre et al., 2014). At present, the microbiota-gut-brain axis is an explanatory model that attempts to relate, among others, ASD symptoms with findings in neuroscience and bacteriology. The microbiota-gut-brain axis is defined as a bi-directional communication system between the neuronal, immune, endocrine and metabolic pathways, but it still requires better understanding (Sudo et al., 2004; Hiippala et al., 2016; Kantarcioglu et al., 2016).

In recent years, there has been an increase in studies indicating a higher prevalence of GI problems in children with ASD compared to healthy children. Specifically, children with ASD have higher rates of diarrhea, constipation and abdominal pain (McElhanon et al., 2014; Krajmalnik-Brown et al., 2015; Martínez-González and Andreo-Martínez, 2019). Thus, children with ASD and with GI problems exhibit more irritability and agitated behavior than children with ASD who do not have GI problems (Rose et al., 2018). On the other hand, some studies indicate that children with ASD present feeding problems. Specifically, they have a greater sensitivity to certain foods or food allergies (Xu et al., 2018) and they have a significantly lower intake of calcium and protein (McElhanon et al., 2014). However, the etiology of the nutritional and GI problems of ASD remains unknown (McElhanon et al., 2014; Ferguson et al., 2016; Martínez-González and Andreo-Martínez, 2019) and everything seems to indicate that it is a combination of associated factors (Buie, 2015). Other authors suggest that abnormal GM in ASD may be caused by the overuse of antibiotics (Sandler et al., 2000; Krajmalnik-Brown et al., 2015; Wimberley et al., 2018) or a pathophysiological cascade of interactions between environmental factors and key genes. In addition, the

neurological alterations associated with atypical growth of the head in children with ASD during the uterine period, which occurs more frequently in late gestations, can also be a possible trigger of ASD (Bonnet-Brilhault et al., 2018).

A recent review up to 2013 reported the different gut microbes involved in ASD (Ding et al., 2017). It seems that higher abundance of *Clostridium* (*Clostridium clostridioforme* or *Clostridium bolteae*) and *Sutterella* genera have been found in children with ASD and with GI problems, while lower levels of *Prevotella* genus have been found in children with ASD. A more recent systematic review up to 2018 reported elevated abundance of *Proteobacteria*, *Lactobacillus*, *Bacteroides*, *Desulfovibrio*, and *Clostridium*, and decreased abundance of *Bifidobacterium*, *Blautia*, *Dialister*, *Prevotella*, *Veillonella*, and *Turicibacter* in children with ASD compared to healthy controls (Liu et al., 2019). There are also other studies and previous reviews in scientific literature about GM and ASD (Wang et al., 2011; Cao et al., 2013; De Angelis et al., 2015; Krajmalnik-Brown et al., 2015; Andreo-Martínez et al., 2019; Martínez-González and Andreo-Martínez, 2019). Although there have been advances in the study of GM in ASD (Ding et al., 2017; Liu et al., 2019), the latest review studies have not been conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2015) within the past six years. Consequently, this study aims to conduct an updated systematic review of the findings of the most common bacterial populations and their abundance in the GM of children with ASD compared to control groups. We expect to find some bacterial population patterns in children with ASD compared to children who do not have this disorder.

The method followed to carry out the present systematic review is described in detail in supporting information, and Figure 1 shows the process of identifying articles for inclusion.

Figure 1

Dysbiosis of the GM in ASD

Table 1 shows the twenty-one articles included in the present systematic review and the results of the analysis of the samples of children with ASD. The gut microbes that showed significantly different abundance in ASD at phylum, family, genus, and specie levels, according to the articles found in the present review, are listed in Figure 2. The phylogenetic tree of this figure was drawn with the help of the Interactive Tree Of Life (iTOL) web-based tool (Letunic and Bork, 2016).

Figure 2

Gut microbiota dysbiosis found in ASD

Nineteen articles found significant differences in some gut microbes' abundance in children with ASD (Table 1) compared to their respective control groups. Specifically, there are 6 bacteria phyla, 16 bacterial families, 1 presumptive bacterial family, 48 bacterial genera, 4 presumptive bacterial genera groups, 1 presumptive bacterial genus and 19 bacterial species of Bacteria domain, and one genus of the Fungi kingdom.

Two studies found no differences in the GM composition of children with ASD (Gondalia et al., 2012; Son et al., 2015). Therefore, the present review expands upon the number of bacteria previously reported (Cao et al., 2013; Ding et al., 2017; Liu et al., 2019). Of note, the recent systematic review published by Liu et al. (2019) reported on different articles from those included in the present systematic review, as they used different inclusion/exclusion criteria such as the inclusion of articles that only analyzed GM by culture-independent methods or the time-frame chosen (from inception to March 2018).In addition, some works have shown contradictory results regarding the nature and/or extent of GM abundance in children with ASD as discussed by Krajmalnik-Brown et al. (2015) and Liu et al. (2019).

The gut microbe abundance of two bacterial phyla, 4 bacterial families, 19 bacterial genera, one genus of the Fungi kingdom and two bacterial species, were found to be significantly different in children with ASD compared to their control groups by more than one article. Among those gut microbes, the abundance of 7 bacterial genera and *Candida* were reported to be similar, while the rest showed abundance discrepancies (Table 1).

The highest number of GM components studied by more than one article was found in Firmicutes phylum (12), followed by Actinobacteria phylum (5), Bacteroidetes phylum (4) and, Proteobacteria phylum (2) and the Deuteromycota phylum (1).

High abundance of some gut microbes in ASD

One bacterial phylum, eight bacterial families, one presumptive bacterial family, thirteen bacterial genera, two presumptive bacterial genera groups, one fungi genus, and

twelve bacterial species were found to have statistically significant higher abundance in children with ASD compared to their control groups. Specifically, at phylum level: Proteobacteria (Coretti et al., 2018) were found to have statistically significant higher abundance in children with ASD compared to their control groups. At family level: Lactobacillaceae, Enterococcaceae, Erysipelotrichaceae and Desulfovibrionaceae (Pulikkan et al., 2018), Peptostreptococcaceae (Berding and Donovan, 2018), Bacteroidaceae, Lachnospiraceae and Ruminococcaceae (Rose et al., 2018), and presumptive Enterobacteriaceae (De Angelis et al., 2013) were found to have statistically significant higher abundance in children with ASD compared to their control groups. At genus level: *Porphyromonas*, presumptive *Bacteroides*, *Porphyromonas* and *Prevotella*, *Caloromator*, *Sarcina*, *Anaerofilum*, presumptive *Pseudomonas* and *Aeromonas*, and *Shigella* (De Angelis et al., 2013), *Ruminococcus* and *Lachnospira* (Coretti et al., 2018), *Collinsella* (Strati et al., 2017), *Mitsuokella* and *Megasphaera* (Pulikkan et al., 2018), *Burkholderia* (Kushak et al., 2017), *Roseburia* (De Angelis et al., 2013; Berding and Donovan, 2018; Coretti et al., 2018), *Peptostreptococcus* and *Ralstonia* (Kushak et al., 2017), and *Candida* (Iovene et al., 2017; Strati et al., 2017) were found to have statistically significant higher abundance in children with ASD compared to their control groups. At species level: *Bilophila wadsworthia*, *Flavonifractor plautii*, *Roseburia intestinalis*, *Lachnoclostridium boltae*, *Lachnoclostridium hathewayi*, *Oscillospira valericigenes*, *Clostridium lituseburense* and *Clostridium aldenense* (Luna et al., 2017), *Ruminococcus gnavus* and *Ruminococcus torques* (Wang et al., 2013), *Lactobacillus ruminis* (Pulikkan et al., 2018), and *Akkermansia muciniphila* (De Angelis et

al., 2013) were found to have statistically significant higher abundance in children with ASD compared to their control groups.

Low abundance of some gut microbes in ASD

Three bacterial phyla, four bacterial families, twenty-two bacterial genera, one presumptive bacterial genus, two presumptive bacterial genera groups, and five bacterial species were found to have statistically significant lower abundance in children with ASD compared to their control groups. Specifically, at phylum level: Fusobacteria, Verrumicrobia (De Angelis et al., 2013), and Actinobacteria (Coretti et al., 2018). At family level: Rikenellaceae (Berding and Donovan, 2018), Actinomycetaceae, Streptococcaceae and Gemellaceae (Coretti et al., 2018). At genus level: *Fusobacterium*, *Eubacterium*, *Subdoligranulum*, *Enterococcus*, *Turicibacter*, presumptive *Staphylococcus*, presumptive *Streptococcus* and *Lactococcus*, and presumptive *Enterococcus* and *Lactobacillus* (De Angelis et al., 2013), *Alistipes* (Strati et al., 2017), *Dialister* and *Bilophila* (Strati et al., 2017; Berding and Donovan, 2018), *Eggerthella* (Coretti et al., 2018), *Peptoniphilus*, *Parvimonas*, *Bulleida* and *Escherichia* (Zhang et al., 2018), *Haemophilus* (Kang et al., 2018), *Neisseria* (Kushak et al., 2017), *Coprococcus* (Kang et al., 2013; Coretti et al., 2018), *Veillonella* (Strati et al., 2017; Zhang et al., 2018), *Prevotella* (De Angelis et al., 2013; Kang et al., 2013; Kushak et al., 2017), *Streptococcus* (De Angelis et al., 2013; Inoue et al., 2016; Kushak et al., 2017; Coretti et al., 2018; Zhang et al., 2018), *Butyricimonas* and *Butyrivibrio* (Berding and

Donovan, 2018), and *Devosia* (Kushak et al., 2017). At species level: *Bacteroides vulgatus*, unidentified *Bacteroides* and *Escherichia coli* (Kushak et al., 2017), *Prevotella copri* and *Haemophilus parainfluenzae* (Kang et al., 2018).

Sample characteristics and other parameters studied

Among the 21 articles found in the present systematic review, none of them studied the communities of viruses and protozoa. Only two articles reported data about Fungi community (Iovene et al., 2017; Strati et al., 2017).

Place where the sample was taken

Three articles analyzed the GM in biopsies taken from the GI tract (Williams et al., 2012; Kushak et al., 2017; Luna et al., 2017), while the rest of the articles analyzed the GM in feces samples.

Bacterial detection methods

Seventeen articles used culture-independent methods (16S rRNA pyrosequencing of bacterial genes or PCR-based detection), three articles used culture-dependent methods (Finegold et al., 2017; Iovene et al., 2017; Gora et al., 2018), and one article used both methods (De Angelis et al., 2013). In this sense, De Angelis et al. (2013) used culture-dependent and culture-independent methods, finding no differences in the abundance of some gut microbes between both methods as reported elsewhere (Alinovi et al., 2009). The case of *Candida* was similar, as higher abundance of this bacterial genus was found in Italian children with ASD using culture-dependent (Iovene et al., 2017) and culture-independent

(Strati et al., 2017) methods. However, in the case of *Lactobacillus* and *Clostridium* the results obtained by different authors using both methods were contradictory.

Among the eighteen articles using culture-independent methods, three used qPCR (Wang et al., 2013; Tomova et al., 2015; Shaaban et al., 2017), three used both qPCR and pyrosequencing of the 16S rRNA gene bacterial (Williams et al., 2012; Kang et al., 2013; Son et al., 2015) and the remaining 12 articles used only pyrosequencing of the 16S rRNA gene bacterial. Among the 15 articles using pyrosequencing of the 16S rRNA gene bacterial method, five used pyrosequencing of the 16S rRNA gene bacterial (V_3-V_4 region) (Inoue et al., 2016; Berding and Donovan, 2018; Coretti et al., 2018; Rose et al., 2018; Zhang et al., 2018), three used pyrosequencing of the 16S rRNA gene bacterial (V_1-V_3 region) (Gondalia et al., 2012; De Angelis et al., 2013; Kushak et al., 2017), two used pyrosequencing of the 16S rRNA gene bacterial (V_2-V_3 region) (Kang et al., 2013; Kang et al., 2018), one used pyrosequencing of the 16S rRNA gene bacterial (V_3-V_5 region) (Strati et al., 2017), one used pyrosequencing of the 16S rRNA gene bacterial (V_1-V_2 and V_1-V_3 region) (Son et al., 2015), one used pyrosequencing of the 16S rRNA gene bacterial (V_1-V_3 and V_4 region) (Luna et al., 2017), one used pyrosequencing of the 16S rRNA gene bacterial (V_2 region) (Williams et al., 2012) and one used pyrosequencing of the 16S rRNA gene bacterial (V_3 region) (Pulikkan et al., 2018). Taking into account the five articles using pyrosequencing of the 16S rRNA gene bacterial (V_3-V_4 region) method, higher abundance of Bacteroidetes and *Roseburia* and lower abundance of *Actinomyces* and *Streptococcus* were found in children with ASD compared to HC by more than one article. However, Firmicutes, Coriobacteriaceae, *Blautia* and

Faecalibacterium showed abundance discrepancies in children with ASD compared to HC in more than one article.

Nature of control groups

Neurotypical (NT) children were used as the control group in four articles (Kang et al., 2013; Luna et al., 2017; Strati et al., 2017; Kang et al., 2018), NT siblings were used as the control group in two articles (Gondalia et al., 2012; Son et al., 2015), both NT siblings and healthy children were used as control groups in one article (Wang et al., 2013), both healthy siblings and healthy children were used as control groups in one article (Tomova et al., 2015), both Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) and healthy siblings were used as control groups in one article (De Angelis et al., 2013), one article used healthy siblings as the control group (Pulikkan et al., 2018), and healthy children were used as the control group in the rest of the articles. Of note, NT children are defined as individuals without neurological, medical, or psychiatric diagnoses (Hamilton et al., 2016), and they can also be referred to as the healthy children group. However, differences in gut microbe abundance can occur when siblings or unrelated people are used as a control group (Krajmalnik-Brown et al., 2015). Any child could be colonized by oro-fecal transmission and, if predisposed with a defective immune system, an abnormal GM related to antimicrobials and other factors such as genetics, could lead to the development of ASD (Finegold et al., 2010).

Nationalities of the children studied

The children with ASD studied were from 9 different nationalities: American (Williams et al., 2012; Kang et al., 2013; Son et al., 2015; Finegold et al., 2017; Kushak et al., 2017; Luna et al., 2017; Berding and Donovan, 2018; Kang et al., 2018; Rose et al., 2018), Australian (Gondalia et al., 2012; Wang et al., 2013), Chinese (Zhang et al., 2018), Egyptian (Shaaban et al., 2017), Indian (Pulikkan et al., 2018), Italian (De Angelis et al., 2013; Iovene et al., 2017; Strati et al., 2017; Coretti et al., 2018), Japanese (Inoue et al., 2016), Polish (Gora et al., 2018) and Slovakian (Tomova et al., 2015). The difference in the nationalities of the children with ASD is also a factor to be taken into account and it is important to consider that there are differences in bacteria prevalence according to the country due to cultural and dietary aspects, among other factors (Lindström and Langenheder, 2012). In this sense, Firmicutes dominate the GM of the general European population (Tomova et al., 2015), and general American and Chinese communities showed high abundances of Firmicutes and Bacteroidetes, respectively (Gupta et al., 2017). In addition, inter-individual and regional differences have been also reported in the general population (Hill et al., 2010; Cerdo et al., 2018) and there is a lack of studies testing the effects of the Mediterranean-style diet on ASD.

Presence of functional gastrointestinal disorders

No functional gastrointestinal disorders (FGID) were reported in five articles (De Angelis et al., 2013; Inoue et al., 2016; Shaaban et al., 2017; Berding and Donovan, 2018; Coretti et al., 2018). Although there is a greater prevalence of GI symptoms in ASD

(McElhanon et al., 2014; Martínez-González and Andreo-Martínez, 2019) there are studies reporting no FGID, as some authors excluded children with ASD and FGID in their studies (De Angelis et al., 2013; Strati et al., 2017; Berding and Donovan, 2018).

Diagnosis of ASD severity and intellectual disability

Two articles reported the ASD severity of the children (Iovene et al., 2017; Pulikkan et al., 2018) and neither of these articles indicated whether children with ASD had been diagnosed with an intellectual disability, which is a very important aspect that influences adaptive ASD behavior (Inada et al., 2015).

Higher or lower abundance of gut microbes in ASD

One bacterial genus (*Roseburia*) and one fungi genus (*Candida*) were found to have statistically significant higher abundance in children with ASD by more than one article (Table 1). *Roseburia* and *Coprococcus* genera (De Angelis et al., 2013) together with some ASD-associated bacteria such as the Clostridia class, and *Bacteroides* and *Desulfovibrio* genera (MacFabe, 2012; MacFabe, 2015) were described as capable of degrading starch and fermenting other carbohydrates to synthesize SCFA. Propionic acid and other SCFAs are mainly produced by active microbiota fermentation of vegetable fiber and/or dietary carbohydrates in the cecum (Nankova et al., 2014; Frye et al., 2016; Rose et al., 2017; Maji et al., 2018). Propionic acid and butyrate can act as colonocytes and mitochondrial fuels, and electrolyte absorption by colon mucosa with anti-inflammatory properties (De Angelis et al., 2013). Propionic acid and butyrate have differential modulatory effects on mitochondrial and

cellular function which can be complex and concentration-dependent (Frye et al., 2015) and can include T-cell function modulation and cytokine production (Rose et al., 2017). Indeed, SCFA can disrupt cellular physiology in order to cause FGID associated with ASD such as non-specific inflammation and dysmotility (Kang et al., 2017; Rose et al., 2017). In addition, butyrate can promote the synthesis of mucin and enhance intestinal tight junction integrity (Zhang et al., 2018). Many genera of the Lachnospiraceae family, such as *Roseburia* and *Dorea,* which were found to have higher abundance in Italian children with ASD, have a very poor capacity to degrade free amino acids (FAAs) (De Angelis et al., 2013). It has been reported that FAAs fecal level increases in children with ASD, especially glutamate (Glu). Some FAAs, particularly Glu, also act as neurotransmitters in the central nervous system, and a Glu excess can lead to neuronal death, so Glu has an important role in the pathophysiology of some neuropsychiatric disorders, including ASD (Won et al., 2012). On the other hand, *Candida albicans* was the most frequently identified *Candida* species in the work of Iovene et al. (2017). In fact, some studies have pointed out that high abundance of *Candida albicans* can cause less carbohydrate and mineral absorption, and generate higher toxin levels that could contribute to ASD behaviors (Kantarcioglu et al., 2016). Other authors hypothesized that some *Lactobacillus* species can modulate the immunological responses to *Candida* in the GI tract by providing tryptophan-derived aryl hydrocarbon receptor ligands that stimulate the immune system, principally IL-C3 cells, to produce IL-22. Together with IL-17, IL-22 prevents the excessive proliferation of *Candida* and other fungal commensals (Zelante et al., 2013; Strati et al., 2017). In other words, IL-22 allows for survival of mixed microbial communities yet provides a pivotal innate antifungal resistance to *Candida* and mucosal

protection from inflammation (Zelante et al., 2013). In this sense, it is possible that ASD dysbiosis may lead to a *Candida* population expansion, preventing complete bacterial community structural restoration, as can be observed by the lower abundance of *Lactobacillus* found in Italian children with ASD and FGID compared to their HC group (Iovene et al., 2017),. However, higher abundance of *Lactobacillus* was also found in Italian children with ASD compared to their NT group (Strati et al., 2017). In conclusion, there is a clear lack of investigation of the whole Fungi kingdom in children with ASD and there is still little known about the influence of *Candida* and other types of fungi on both GI and ASD symptoms (Andreo-Martínez et al., 2019).

In addition, six bacterial genera (*Prevotella*, *Dialister*, *Bilophila*, *Veillonella*, *Streptococcus* and *Coprococcus*) were found by more than one article to have statistically significant lower abundance in children with ASD (Table 1).

Lower abundance of *Prevotella* has been found in feces of Italian children with ASD compared to their sibling group (De Angelis et al., 2013), in feces of American children with ASD compared to their NT group (Kang et al., 2013), and in duodenal biopsy samples from the second part of the duodenum of American children with ASD compared to their HC group (Kushak et al., 2017). As can be observed, similar results were achieved by comparing two different nationalities, two different control groups and two different places where the sample was taken.

It is believed that the presence of *Prevotella* and other Bacteroidetes are associated with colon health and ASD manifestations may have their origin in a dysbiosis where *Prevotella* decreases and *Sutterella* increases (Ding et al., 2017). In this sense, the lower

abundance of *Prevotella* found in American children with ASD was associated with the presence of ASD symptoms instead of FGID (Kang et al., 2013). These authors found no correlation in a later work (Kang et al., 2018), although they found that FGID were significantly more severe in American children with ASD compared to their NT group. As *Prevotella* is just one genus of the GM that is very diverse and has a tendency to coexist with a complex collection of other bacterial species, Krajmalnik-Brown et al. (2015) tried to find an explanation about the lower abundance of *Prevotella* in American children with ASD compared to both Malawian and Venezuelan children with ASD. They found that *Prevotella* may act as an indicator of Westernization and this change could also be framing an altered immune system, but their results were not conclusive. Finally, *Desulfovibrio* works synergistically with *Prevotella* to degrade mucin and *Desulfovibrio*, *Prevotella*, and *Oscillibacter* can utilize microbial exopolysaccharides synthesized by *Bifidobacterium* to produce SCFA in the GI tract (Kang et al., 2013). In this sense, the lower abundance of *Prevotella* can decrease the SCFA level in the GI tract of children with ASD.

Veillonella was less abundant in Chinese (Zhang et al., 2018) and Italian (Strati et al., 2017) children with ASD compared to their HC and NT groups, respectively (Table 1). Different nationalities and similar control groups showed no differences in this bacteria genus. In addition, *Coprococcus*, Veillonellaceae and Enterococcaceae, together with *Prevotella*, are intriguingly versatile bacteria that degrade and/or ferment carbohydrates, suggesting that differences in microbial composition (especially in *Veillonella*) of children with ASD may be a consequence of diet (Kang et al., 2013; Pulikkan et al., 2018). Then, a lower abundance of *Veillonella* may disturb the fermentation of lactate in children with ASD,

as this bacterial genus is capable of fermenting lactate (Zhang et al., 2018). As *Dialister* is a bacteria belonging to the Veillonellaceae family, the aforementioned statement for *Veillonella* could be also valid for *Dialister*, as this bacterial genus also showed lower abundance in American (Berding and Donovan, 2018) and Italian (Strati et al., 2017) children with ASD compared to HC. Further, lower abundance of *Dialister* has been statically correlated with nutritional intake. Specifically, children with ASD eating 20 foods or less in their diet had lower intakes of pectin, vitamin C, niacin, vitamin B6, folate, and selenium, but higher intakes of added sugars (Berding and Donovan, 2018). *Streptococcus* was found to be less abundant in duodenal biopsy samples from the second part of the duodenum of American children with ASD compared to their HC group (Kushak et al., 2017), in feces of Chinese (Zhang et al., 2018) and Japanese (Inoue et al., 2016) children with ASD compared to their HC groups, respectively, and in feces of Italian children with ASD compared to their HC (Coretti et al., 2018) and siblings (De Angelis et al., 2013) groups, respectively, with *Streptococcus* and *Enterococcus* being the dominant genera of the Bacilli class in the work of De Angelis et al. (2013). Those results are similar even when comparing four different nationalities, two different control groups and two different places where the sample was taken.

Streptococcus together with *Lactobacillus*, *Bifidobacterium* and *Lactococcus* produce lactate (Zhang et al., 2018). It is known that ASD patients show high levels of lactate (Rossignol and Frye, 2011), suggesting an increase in glycolysis through the phenomenon of aerobic glycolysis in ASD since the dysregulation of this balance has been proposed as a potential cause of ASD (Vallée and Vallée, 2018). At this point, we cannot confirm that the

increase in lactate in people with ASD can be attributed solely to the GM since, as seen below, there is still no consensus regarding the abundance of certain lactate-producing bacteria such as *Lactobacillus* or *Bifidobacterium*.

American children with ASD compared to their NT group (Kang et al., 2013) and Italian children with ASD compared to their HC group (Coretti et al., 2018) showed lower abundance of *Coprococcus* (Table 1). In this case, two different nationalities and similar control groups coincided in low abundances of this bacterial genus.

Coprococcus comes was positively correlated with aerophagia and *Coprococcus catus* was negatively correlated with abdominal migraine (Luna et al., 2017), showing the role played by some *Coprococcus* species in FGID. Finally, as discussed before, *Coprococcus* also produces SCFA, suggesting that the unusual diet patterns observed in ASD children may be a possible influence (Kang et al., 2013).

Bilophila is a sulfite-reducing and hydrogen sulfide-producing bacteria in the human GI tract which is usually difficult to detect in healthy individuals. Increased numbers of *Bilophila* in GM induce systemic inflammation and thus contributes to the onset of the metabolic disorders (Feng et al., 2017). The present systematic review found lower abundance of *Bilophila* in American (Berding and Donovan, 2018) and Italian (Strati et al., 2017) children with ASD compared to HC, therefore, further research is needed regarding the implications of this bacterial genus in ASD. Regarding the Bacteroidetes/Firmicutes ratio in the GI tract of children with ASD, it is characterized by a lower Bacteroidetes/Firmicutes ratio in Italian, Slovakian, and Indian children with ASD compared to their heterogenous control groups (De Angelis et al., 2013; Tomova et al., 2015; Strati et al., 2017; Pulikkan et

al., 2018). However, according to Coretti et al. (2018) and Zhang et al. (2018), the Bacteroidetes/Firmicutes ratio in Italian and Chinese children with ASD, respectively, was higher than their HC groups. As discussed before, different nationalities, control groups, the place where the sample was taken or bacterial detection methods can lead to contradictory results. In addition, Tomova et al. (2015) hypothesized that the possible reasons for contradictory results in different studies can be due to participants' age (younger people's GM contain proportionally fewer Bacteroidetes), GI problems in HC, GI tract level where sample was taken, or geographical area. Therefore, for the correct interpretation of GM's role in ASD, etiopathogenesis can be important to compare matching homogeneous groups.

Possible reasons of the differences found in GM of ASD

The differences found in GM of children with ASD can be attributed to ASD heterology, small sample sizes, different places where the sample were taken, different bacterial identification methods, different nationalities, inter-individual differences, antibiotic use, malnutrition, dietary habits, age of participants and nature of control groups, prebiotics and probiotics used, and FGID presence, among others. For example, it has been reported that GM communities of fecal samples are similar to luminal communities; however, significantly higher abundances of some gut microbes that preferentially adhere to the mucosa, such as *Lactobacillus*, compared to fecal samples have been found. Therefore, the place where the sample were taken can be relevant for mammalian physiology, as bacterial communities display differences between anatomical locations (Hill et al., 2010; Durbán et al., 2012; Martínez-González and Andreo-Martínez, 2019). Regarding bacterial detection methods: on

the one hand, absolute cell abundances of culturable living microbes can be quantified using culture-dependent methods, although the number of microorganisms detected is limited and this method requires some days to obtain results. On the other hand, the use of culture-independent methods has increased in recent years due to the fact that they can readily identify a large proportion of the bacterial diversity and they give fast results. In addition, amplification bias in heterogeneous mixtures may occur due to differences in genomic sequences at primer sites, 16S rRNA copy number, different bioinformatic analysis methods and guanine-cytosine content, such that the read counts can be correlated semi-quantitatively with the relative abundance of bacterial species (Gondalia et al., 2012; Salipante et al., 2013; Zapka et al., 2017). Therefore, future studies should use similar bacterial detection methods in order to minimize these biases, taking into account the fact that culture-independent methods are relatively new in comparison with culture-dependent methods and they are still developing and not yet standardized (Zapka et al., 2017). Thus, future microbial technological developments should combine omics data with phenotypic information to invoke and control specific phenotypes in the microbiome (De Vrieze and Boon, 2018). Of note, there are still discrepancies in the abundance of some bacteria using the same bacterial detection method, as discussed in section 3.2.2. In addition, one explanation regarding the differences in gut microbe's abundance when siblings or unrelated people are used as a control group could be that the children of both samples intake a similar diet, or the possible fecal bacteria transmission from children with ASD to their siblings (multiple ASD cases are common in families), playmates, friends, etc. resulting in similar bacterial abundance (Tomova et al., 2015).

Concluding remarks

At this point, there is no consensus about a GM profile of children with ASD and further research is needed. In addition, an important absence in the psychometric analysis of the relationship between the severity of behavioral ASD symptoms and bacterial abundance and FGID has been found. Similarly, none of the studies indicate whether children with ASD have been diagnosed with an intellectual disability.

Therefore, the results of the present systematic review encourage the need to initiate new multicenter studies on the impact of bacterial components of GM on: gastrointestinal physiology, neurophysiology, metabolomic and behavior of children with ASD. We consider that ASD has a wide phenotypic variability, so future studies should consider the genetic, phenotypic and behavioral antecedents in a more integral and interdisciplinary way. In addition, we consider it of great importance that future studies classify ASD groups according to the severity of ASD and intellectual disability. Finally, we argue that future research should homogenize statistical analysis in order to develop meta-analytical studies on the GM involved in ASD.

Author Contributions

Pedro Andreo-Martínez and Agustín Ernesto Martínez-González conceived and designed the systematic review. Pedro Andreo-Martínez and Nuria Garcia-Martínez carried out the

systematic review process. Pedro Andreo-Martínez, Nuria García-Martínez and Elvira Pilar Sánchez-Samper wrote the manuscript. Agustín Ernesto Martínez-González edited the manuscript and all the authors approved the final version of the manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Compliance with Ethical Standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors would like to acknowledge Ms. Seonaid McNabb and Mr. Andy McCarthy for their English revision. We appreciate the initial contact and kindness of Dr. Derrick MacFabe (Kilee Patchell-Evans Autism Research Group, London, Ontario, Canada).

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Table, Figure and supporting information legends

Figure 1. Flowchart showing the process of identifying relevant studies for the present systematic review.

Figure 2. Phylogenetic map of bacterial populations (Phyla, families, genera and species level) in the ASD.

Note: [A = Williams et al. (2012); B = Gondalia et al. (2012); C = Kang et al. (2013); D = Wang et al. (2013); E = De Angelis et al. (2013); F = Son et al. (2015); G =Tomova et al. (2015); H = Inoue et al. (2016); I = Strati et al. (2017); J = Iovene et al. (2017); K= Luna et al. (2017); L = Finegold et al. (2017); M = Kushak et al. (2017); N = Shaaban et al. (2017); O = Gora et al. (2018); P = Kang et al. (2018); Q = Pulikkan et al. (2018); R = Rose et al. (2018); S = Coretti et al. (2018); T = Zhang et al. (2018); U = Berding and Donovan (2018)].

Table 1. Results of selected articles on ASD and gut microbiota.

Supporting information. Method

Figure 1

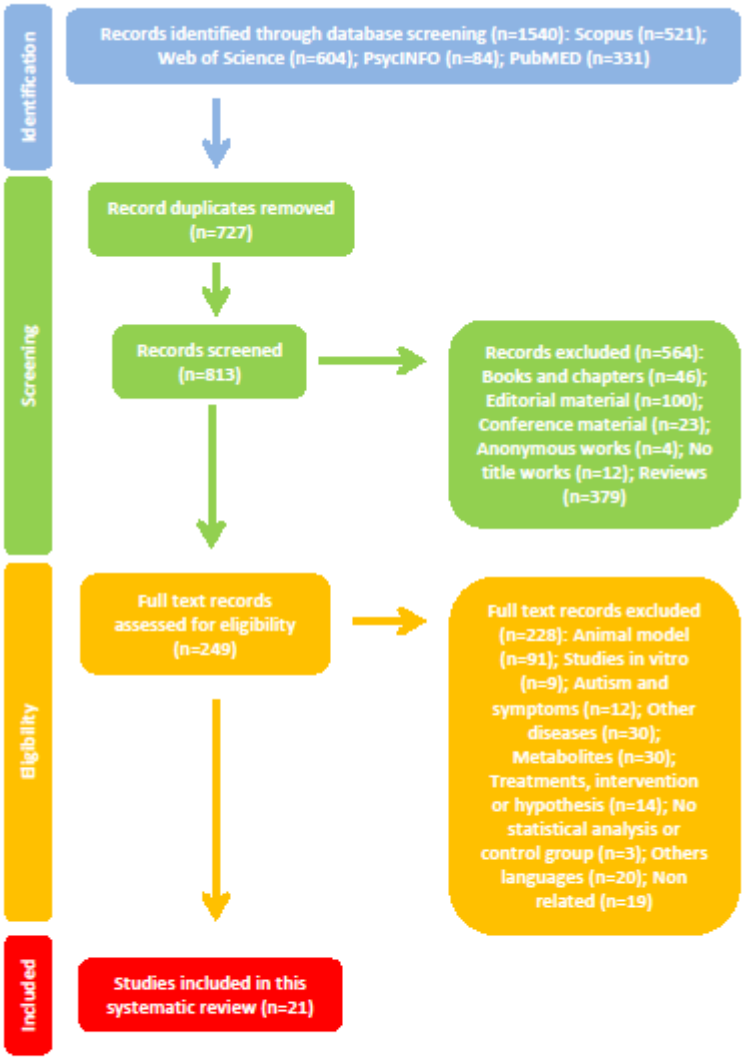

Figure 2

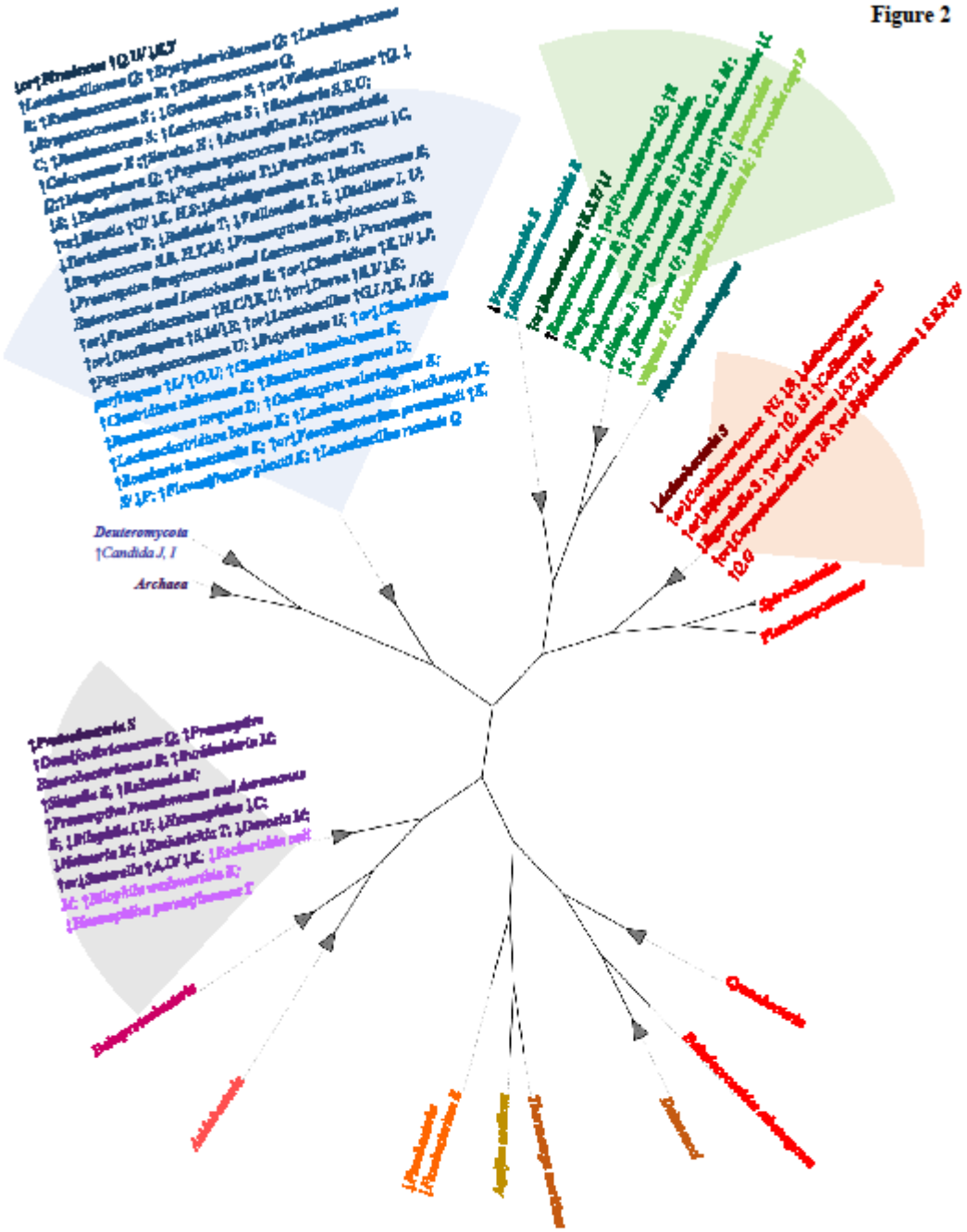

Table 1. Results of selected studies on ASD and gut microbiota.

Ref	Subjects		Method	Findings		Limitations
	Experimental group	**Control group**		**Bacteria**	**Correlation**	
Williams et al. (2012)	ASD (n=23) divided into ASD w FGID (12/23) and ASD w/o FGID (11/23) Range Age 3-10 y (15/23 aged 3-5 y; 6/23 aged 6-7 y; 2/23 aged 8-10 y) All males	HC w FGID (n=9) Range Age 3-10 y (7/9 aged 3-5 y; 1/9 aged 6-7 y; 1/9 aged 8-10 y). All males	Pyrosequencing of the 16S rRNA gene pan-bacterial (V2 region) on ileal and ceca mucosal biopsy samples PCR-based detection of *Sutterella* 16S rRNA gene sequences (V6–V8 region and C4–V8 region) on ileal and ceca mucosal biopsy samples Western immunoblots for soluble proteins of cultured *Sutterella wadsworthensis*	*Sutterella* ↑ in ASD w FGID *vs* HC w FGID (ilea p=0.022 / ceca p=0.037)	Plasma IgG or IgM antibody reactivity to *Sutterella* antigens in ASD w FGID	ASD severity is not indicated It is not indicated whether the ASD have intellectual disability
Gondalia et al. (2012)	ASD (n=51) divided into ASD w FGID (28/51) and ASD w/o FGID (23/51) Age 2-12 y 42 males 9 females	NT Sib (n=53) Age 2-12 y 19 males 34 females	Pyrosequencing **(**bTEFAP**)** of the 16S rRNA gene bacterial (V1-V3 regions) on fecal samples	No difference in microbiome between total ASD *vs* NT Sib. No difference regarding severity ASD in the bacterial composition. No difference regarding GI dysfunction in the bacterial composition (No differences within the ASD group compared to both with and without FGID groups)	No metabolites were studied	It is not indicated whether the ASD have intellectual disability The dietary intake was not controlled
Kang et al. (2013)	ASD w FGID (n=20) Age 3-16 y 18 males 2 females	NT w FGID (n=20) Age 3-16 y 17 males 3 females	Pyrosequencing **(**bTEFAP**)** of the 16S rRNA gene bacterial (V2-V3 regions) on fecal samples Quantitative real-time PCR for *Prevotella*	*Prevotella* (p=0.04), *Coprococcus* ($p \leq 0.06$) and unclassified *Veillonellaceae* (p=0.04) ↓ in ASD *vs* NT. Microbial changes were more closely linked to the presence of autistic symptoms rather than to the severity of GI symptoms and specific diet/supplement regimens	No difference between GI severity problems and ASD severity indexes	It is not indicated whether the ASD have intellectual disability
Wang et	ASD (n=23)	HC (n=9)	qPCR for	*Sutterella* ↑ (p=0.044) in	No metabolites were	ASD severity is

al. (2013)	w or w/o FGID Age 3-18 y Unrevealed sex	and NT Sib (n=22) w or w/o FGID Age 3-18 y Unrevealed sex	*Sutterella*, *Ruminococcus torques* and *R. gnavus* on fecal samples.	ASD *vs* NT Sib. *Sutterella* ↑ (p=0.05) in ASD *vs* HC. *Sutterella* ↑ (p=0.047) in NT Sib *vs* HC. *Ruminococcus gnavus* ↑ (p=0.046) in NT Sib *vs* HC. *Ruminococcus torques* ↑ (p=0.008) in ASD w FGID (n=9) *vs* ASD w/o FGID (n=14).	studied	not indicated It is not indicated whether the ASD have intellectual disability The dietary intake was not controlled GI symptoms were not correlated
De Angelis et al. (2013)	ASD (n=10) PDD-NOS (n=10) Age 4-10 y Unidentified sex	HC sibling (n=10) Age 4-10 y Unidentified sex	Pyrosequencing **(**bTEFAP**)** of the 16S rRNA gene bacterial (V1-V3 regions) on fecal samples Selective culture methods for bacterial species on fecal samples: PCA (total facultative aerobes and anaerobes); MRS agar (L*actobacilli* and E*nterococci*); *Bifidobacterium* agar modified (*Bifidobacteria*); M17 (*Lactococci* and S*treptococci*); Mannitol salt agar (*Staphylococci*); Wilkins-Chalgren anaerobe agar (total anaerobes); Wilkins-Chalgren anaerobe agar + GN and sheep blood (*Bacteroides*, *Porphyromonas* and *Prevotella*); Reinforced	**Pyrosequencing data:** Gut microbial diversity ↑ in ASD and PDD-NOS *vs* HC Bacteroidetes ↑, Firmicutes ↓, Fusobacteria ↓, Verrumicrobia ↓ in ASD *vs* HC (p<0.05) *Faecalibacterium* ↓ in ASD *vs* HC. *Anaerofilum* ↑, *Oscillospira* ↓, *Subdoligranulum* ↓ in ASD *vs* HC (p<0.05) *Clostridium* ↑, in ASD *vs* HC (p<0.05) *Caloramator* and *Sarcina* ↑ in ASD *vs* HC (p<0.05) *Roseburia* ↑ (p<0.05). *Dorea* ↑ in ASD *vs* HC (p<0.05) *Eubacterium* ↓ in ASD *vs* HC (p<0.05) *Streptococcus* ↓ in ASD *vs* HC (p<0.05)	*Clostridium* with methyl esters (butanoic acid methyl ester, acetic acid methyl ester and pentanoic acid methyl ester) and indoles (r=1.0; p<0.05) *Faecalibacterium*, *Ruminococcus* and *Bifidobacterium* with total SCFAs (r=1.0; p<0.05) *Bacteroides* with total FAA, NH_3 and PPA (r=1.0; p<0.05)	ASD severity is not indicated It is not indicated whether the ASD have intellectual disability GI symptoms were not correlated

	Clostridial Medium + novobiocin and colistin (*Clostridium*); MacConkey agar Nº2 (*Enterobacteria*); Rogosa agar + glacial acetic acid (*Lactobacilli*); GSP agar + penicillin-G (*Pseudomonas*, *Aeromonas*); and S&B (*Enterococci*)	*Turicibacter* ↓ in ASD *vs* HC (p<0.05) *Bacteroides* ↑ in ASD *vs* HC (p<0.05) *Prevotella* ↓ in ASD *vs* HC (p<0.05) *Shigella* ↑ in ASD *vs* HC (p<0.05) *Bifidobacterium* ↓ in ASD *vs* HC (p<0.05) *Fusobacterium* ↓ in ASD *vs* HC (p<0.05)
	Biochrom 30 series amino acid analyzer for concentration of FAA in feces	*Akkermansia muciniphila* ↑ in ASD *vs* HC (p<0.05)
		Cultivable bacteria data:
	Gas-chromatography mass spectrometry-solid-phase microextraction (GC-MS/SPME) analysis of fecal volatile compounds	No differences between PDD-NOS, ASD and HC for total microbes Total anaerobes were the highest in HC (p<0.05) *Enterococcus*, *Lactobacillus*, *Streptococcus*, Presumptive Streptococcus and *Lactococcus*, presumptive *Enterococcus and Lactobacillus*, *Staphylococcus* ↓ in ASD *vs* HC (p<0.05) *Bifidobacterium* ↓ in ASD *vs* HC and PDD-NOS (p<0.05) *Clostridium*; Presumptive *Bacteroides*, *Porphyromonas* and *Prevotella*; Presumptive *Pseudomonas* and*Aeromonas;* and Enterobacteriaceae ↑ in ASD *vs* HC (p<0.05)

Son et al. (2015)	ASD (n=59) divided into ASD w FGID (25/59) and ASD w/o FGID (34/59) Age 7-14 y 52 males 7 females	NT Sib (n=44) divided into NT Sib w FGID (13/44) and NT Sib w/o FGID (31/44) Age 7-14 y 21 males 23 females	qPCR for total bacteria, *Sutterella* subgroup, *Bacteroidetes* subgroup, *Prevotella*, *C. coccoides-E. rectales* subgroup, *Faecalibacterium prausnitzii* and *Escherichia coli* subgroup on fecal samples Sequencing of the 16S rRNA gene bacterial (V1-V2 and V1-V3 regions) on fecal samples	No difference between total ASD *vs* NT Sib in bacterial frequency. Increased prevalence of functional constipation FGID in ASD children compared to NT siblings	Increased prevalence of functional constipation FGID in ASD children compared to NT siblings.	ASD severity is not indicated Is not indicated whether the ASD have intellectual disability
Tomova et al. (2015)	ASD (n=10) divided into ASD w FGID (9/10) and ASD w/o FGID (1/10) Age 2-9, 9 males and 1 female Sib (n=9) divided into Sib w FGID (7/9) and Sib w/o FGID (2/9) Age 5-17, 7 males and 3 females	HC (n=10) divided into HC w FGID (6/10) and HC w/o FGID (4/10) Age 2-11, 10 males	qPCR for Bacteroidetes, Firmicutes, *Bifidobacterium*, *Lactobacillus*, *Clostridium* cluster 1, *S. thermophiles*, *Desulfovibrio* on fecal samples ELISA for TNF-α on the stool ELISA for oxytocin, testosterone and dehydroepiandrosterone-sulfate (DHEA-S) levels on plasma	Bacteroidetes/Firmicutes ratio↓ in ASD *vs* HC (p<0.05); ↓ Sib *vs* ASD (p<0.05); ↓ Sib *vs* HC (p<0.05) Firmicutes ↑ in Sib *vs* HC *Lactobacillus* ↑ in ASD *vs* HC (p<0.05) *Bifidobacterium* ↑ in ASD *vs* Sib (p<0.05)	*Desulfovibrio* with ASD severity in the ADI restricted/repetitive behavior subscale score. No correlation between plasma levels of oxytocin, testosterone, DHEA-S and gut microbiota	It is not indicated whether the ASD have intellectual disability The dietary intake was not controlled
Inoue et al. (2016)	ASD (n=6) Age 3-5 y Unrevealed sex	HC (n=6) Age 3-5 y Unrevealed sex	Sequencing of the 16S rRNA gene bacterial (V3-V4 regions) on fecal samples Gene expression of PBMC by microarray analysis, on blood samples RT-PCR for IFN-related genes	*Blautia* ↓ in ASD *vs* HC (p<0.05) *Faecalibacterium* ↑ in ASD *vs* HC (p<0.05) *Streptococcus* ↓ in ASD vs HC	↑*Faecalibacterium* and a greater number of differentially expressed genes involved in both the interferon (IFN)-γ-mediated signaling pathway and the type I interferon signaling pathway, (in particular in the latter)	ASD severity is not indicated It is not indicated whether the ASD have intellectual disability Small number of participants Dietary intake was not controlled

			(IRF7, IRF9, CXCL10 and CXCL11) in PBMC			
Strati et al. (2017)	ASD (n = 40) divided into severe ASD (36/40) and moderately severe ASD (4/40) 5 constipated 29 non-constipated Average age 11.1±6.8 31 males 9 females	NT (n=40) 11 constipated 29 non-constipated Average age 9.2±7.9 28 males 12 females	Pyrosequencing of the 16S rRNA gene bacterial (V3-V5 regions) and the internal transcribed spacer (ITS) for fungal (ITS1 rDNA region) on fecal samples ELISA for calprotectin determination on fecal samples	Firmicutes/Bacteroidetes ratio↑, *Bacteroidetes* ↓, *Veillonella* ↓, *Alistipes* ↓, *Bilophila*↓, *Dialister*↓, *Parabacteroides*↓ in ASD *vs* NT (p<0.005) *Lactobacillus* ↑, *Dorea* ↑, *Corynebacterium* ↑, *Collinsella* ↑, *Candida* ↑ in ASD *vs* NT (p<0.001)	*Escherichia*/*Shigella* and cluster XVIII with GI symptoms or constipation (p<0.05)	It is not indicated whether the ASD have intellectual disability It should be noted that the number of enrolled constipated subjects was quite low and therefore these analyses could be underpowered
Iovene et al. (2017)	Severe ASD (n=47) w/o or w FGID Average age 6.0±2.8 y 40 males 7 females	HC (n=33) w/o or w FGID Average age 7.3±3.1 y 24 males 9 females	Examination and culture of fecal samples: (a) morphological examination, (b) microscopic examination staining, (c) search for toxins a/b of *Clostridium difficilis*, (d) bacterial/yeast culture and (e) identification of bacteria and yeast colonies by VITEK 2 microbial identification system ELISA for calprotectin determination on fecal samples Lactulose/mannitol (LA/MA) test for Intestinal permeability (IP)	*Candida* ↑ in ASD *vs* HC (p=8.67x10^{-6}) *Lactobacillus* ↓ in ASD *vs* HC (p=7.28x10^{-4}) *Clostridium* ↓ in ASD *vs* HC (p=0.01)	Leukocytes ↑ in ASD 14.89% (7/47) vs HC (p=0.01) *Clostridium* and calprotectin with CARs (p=0.03) Lactulose ↑ in ASD μ_1=0.550 *vs* HC μ_2=0.249 (p=0.03)	It is not indicated whether the ASD have intellectual disability The dietary intake was not controlled GI symptoms were not correlated
Luna et al. (2017)	ASD w FGID (n=14)	NT w FGID (n=15)	Sequencing of the 16S rRNA gene (V1-V3 and V4 regions) on	*Blautia* ↓ (p=0.02), *Dorea* ↓ (p=0.006), *Sutterella* ↓ (p=0.025) in ASD w FGID *vs* NT w	IBS with ↑ *Clostridium aldenense* (p=0.04); Functional constipation	ASD severity is not indicated Is not indicated

	Age 4-13 **y** 14 males	Age 3-18 y 12 males 3 females NT w/o FGID (n=6) Age 3-18 y 6 males	rectum mucosal biopsy samples 38 cytokines by the MagPix system on biopsy rectum and blood Serotonin, 5-HIAA and Tryptophan by high-performance liquid chromatography on biopsy rectum	FGID *Clostridium lituseburense* ↑ (p=0.002), *Lachnoclostridium bolteae* ↑ (p=0.017), *Lachnoclostridium hathewayi* ↑ (p=0.03), *Clostridium aldenense* ↑ (p=0.03), and *Flavonifractor plautii* ↑ (p=0.03), in ASD w FGID *vs* NT w FGID and NT w/o FGID *Faecalibacterium prausnitzii* ↑, *Roseburia intestinalis* ↑, *Oscillospira valericigenes* ↑, and *Bilophila wadsworthia* ↑ (p<0.05) in NT w FGID *vs* NT w/o FGID	with ↓ *Flavonifractor plautii* (p=0.03), *Bacteroides eggerthii* (p=0.02), *Bacteroides uniformis* (p=0.04), *Faecalibacterium prausnitzii* (p=0.013), *Clostridium clariflavum* (p=0.03); Aerophagia with ↑ *Clostridium aldenense* (p=0.03), ↓ in *Blautia luti* (p=0.003), *Bifidobacterium adolescentis* (p=0.01), *Eubacterium ventriosum* (p=0.05), *Anoxystipes fissicatena* (p=0.02), *Coprococcus comes* (p=0.04), *Eubacterium ramulus* (p=0.006), and *Phascolarctobacterium faecium* (p=0.04); Abdominal migraine with ↓ in *Akkermansia muciniphila* (p=0.03), *Coprococcus catus* (p=0.007), *Odoribacter splanchnicus* (p=0.05), *Clostridium lactatifermentans* (p=0.03) and *Ruminococcus lactaris* (p=0.03) Serotonin with *Lachnoclostridium bolteae* (p=0.002), *Lachnoclostridium hathewayi* (p=0.003) and *Flavonifractor plautii* (p=0.001)	whether the ASD have intellectual disability The dietary intake was not controlled
Finegold et al. (2017)	ASD w FGID (n=33) Age 2-9 y Unidentified sex	HC w/o FGID (n=13) Age 2-9 y Unidentifie d sex	Selective culture methods for *Clostridium* and for *Clostridium perfringens* strains on fecal samples: Brucella and CDC agar PCR for *Clostridium perfringens* toxin genes: alpha (cpa), beta (cpb),	*Clostridium perfringens* ↑ in ASD w FGID *vs* HC (p=0.03)	↑ counts of Beta 2-Toxin gene-positive *Clostridium perfringens* in ASD *vs* HC (p=0.01) ↑ incidence of Beta 2-Toxin *Clostridium perfringens* in ASD *vs* HC (p=0.01)	ASD severity is not indicated It is not indicated whether the ASD have intellectual disability The dietary intake was not controlled

			beta 2 (cpb2), epsilon (ctx), iota (iA) and enterotoxin (cpe)			
Kushak et al. (2017)	ASD (n=21) w FGID Age 14.4±1.1 y 19 males 2 females	HC (n=19) w FGID Age 16±1.2 y 10 males 9 females	Pyrosequencing of the 16S rRNA gene bacterial (V1-V3 regions) on duodenal biopsies samples from the second part of the duodenum Dahlqvist method for analysis of disaccharidase activity: lactase, sucrose, maltase and palatinase Bradford method for protein content	No differences in microbiome diversity (alpha and beta) *Burkholderia* ↑ (p=0.03) and *Neisseria* ↓ (p=0.01) in ASD *vs* HC *Bacteroides vulgatus* ↓ (p=0.005), unidentified *Bacteroides* ↓ (p=0.04) and *Escherichia coli* ↓ (p=0.05) in ASD *vs* HC *Oscillospira*, *Actinomyces*, *Peptostreptococcus* and *Ralstonia* ↑ (p<0.05) in ASD *vs* HC *Devosia*, *Prevotella*, *Bacteroides* and *Streptococcus* ↓ (p<0.05) in ASD *vs* HC No differences in disaccharidase activity between groups	↑ frequency of constipation in ASD *vs* HC (p<0.005) *Bacteroides* and *Faecalibacterium* showed a strong positive correlation with lactase activity in ASD group *Clostridium* had a strong positive correlation with disaccharidase activity (lactase, maltase, palatinase and sucrose) in ASD group *Porphyromonas*, *Barnesiella*, *Gemella*, and *Leptotrichia* had a strong positive correlation with lactase activity in HC group	ASD severity is not indicated Is not indicated whether the ASD have intellectual disability Heterogeneity in age and sex in study population and presence of various FGID in ASD and HC children. The role of diet and particularly dietary restrictions in ASD group remains a confusing factor in data analysis

Shaaban et al. (2017)	ASD (n=30) ASD (n=30) supplemented with *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacteria longum*, during 3 months. Age 5-9 y 19 males 11 females	HC parients (n=30) Age 5-9 y Unrevealed sex	Quantitative real-time PCR in stool samples	*Bifidobacterium* ↓ in ASD *vs* HC (p=0.0001) After probiotic supplementation: *Bifidobacterium* ↑ and *Lactobacillus* ↑ in supplemented-ASD *vs* ASD (p<0.0001) Body weight ↓ in supplemented-ASD *vs* ASD (p=0.014) Severity of ASD symptoms ↓ (p=0.0001) and FGID ↓ (p<0.05) in supplemented-ASD *vs* ASD	*Bifidobacterium* negatively correlated with the reduction constipation after probiotics use (r=−0.441, p<0.015) Improvements in FGID correlated positively strongly with improvements in the severity of ASD symptoms after probiotic use (r=0.674, p=0.0001) ↑ *Bifidobacterium* correlated negatively with decreased of body weight (p=0.044) and body weight z-score (p=0.033) after probiotic use ↑ *Lactobacilli* correlated positively with the decrease in body weight (p=0.006) and the decreased of BMI (p=0.003) after probiotic use	ASD severity is not indicated It is not indicated whether the ASD have intellectual disability A small sample size, unblinding and the lack of a placebo control group The cohort received behavioral therapy during the probiotic's administration.
Gora et al. (2018)	ASD (n=29) w FGID (49 isolated strains of *Clostridium perfringens*) Age 3.5-18 y 23 males 9 females	HC (n=17) (30 isolated strains of *Clostridium perfringens*) Obese children (n=24) (32 isolated strains of *Clostridium perfringens*) Unrevealed age and sex	Selective culture method for fecal samples: Columbia blood and Reinforced Clostridial agar under anaerobic conditions. Hemolysis test, lecithinase and lipase production on Egg-Yolk-Agar, and identified with use of ANC cards in VITEK 2 compact. Subcultured in BHI broth and GeneMATRIX DNA Purifi-cation Kit by DNA Gdansk for isolation of DN Multiplex PCR for toxin alpha (*cpa*),	The *cpa* gene encoding alpha toxin was present in all 111 (100%) strains The *cpb2* gene encoding beta2 toxin was found in: 45/49 (91.8%) strains isolated from ASD children, 17/30 (56.7%) strains isolated from healthy subjects (p<0.001), and 12/32 (37.5%) strains isolated from obese children (p<0.001) *Clostridium perfringens* strains with the ability to produce beta2 toxin (*cpb2* gene) were detected in: 27/29 (93.1%) ASD, 10/17 (58.8%) HC (p<0.008), and 11/24 (45.8%) obese children (p<0.001) No differences between HC and obese children (*cpb2* and *Clostridium perfringens* with *cpb2*)		ASD severity is not indicated. Is not indicated whether the ASD have intellectual disability A small number of studied patients and strains

			toxin beta (*cbp*), enterotoxin (*ecpe*), iotatoxin (*cpiA*), epsilon toxin (*etx*) genes			
Kang et al. (2018)	ASD (n=23) divided into ASD w FGID (21/23) and ASD w/o FGID (2/23) Age 4-17 y 15 males 6 females	NT (n=21) divided into NT w FGID (10/21) and NT w/o FGID (11/21) Age 4-17 y 22 males 1 female	Pyrosequencing of the 16S rRNA gene bacterial (V2-V3 regions) on fecal samples Metabolite profiles by ^{1}H-NMR spectroscopy in fecal samples	Gut microbial diversity (alpha) ↓ (p<0.001) and relative abundances of phylotypes most closely related to *Prevotella copri* ↓ (p<0.04) in ASD *Faecalibacterium* ↓ (p<0.01) and *Haemophilus* ↓ (p<0.05) in ASD *vs* NT *Feacalibacterium prausnitzii* ↓ (p<0.01) and *Haemophilus parainfluenzae* ↓ (p<0.05) in ASD *vs* NT	Isopropanol ↑ in ASD 82% *vs* NT (p=0.022) p-cresol ↑ in ASD 26% *vs* NT (p=0.04) *GABA* trends ↓ in ASD (36%) *vs* NT (p=0.077) p-cresol concentrations were significantly and negatively correlated with age within the ASD group (Spearman correlation, r=0.47, p=0.02) GI symptoms were significantly more severe for children with ASD compared to controls No correlation between all bacterial taxa and metabolites	ASD severity is not indicated. Is not indicated whether the ASD have intellectual disability Genomic database limitations: isopropanol producing and degrading enzymes could not be investigated) A lack of meaningful observations between fecal metabolites and microbiome profiles using PICRUSt may be attributed to a difference in dataset types, a small sample size, and statistical tools
Pulikkan et al. (2018)	ASD (n=30) divided into severe ASD (28/30) and moderate ASD (2/30) w FGID Age 3-16 y 28 males 2 females BMI 6.9-20.5	HC mostly Sib or blood relatives to the ASD children (n=24) w/o FGID Age 3.5-16 y 15 males 9 females BMI 13.4-31	Sequencing of the 16S rRNA gene bacterial (V3 region) on fecal samples	Firmicutes ↑ in ASD *vs* HC Sib (p<0.05) Prevotellaceae ↓ and Veillonelleaceae ↑ in ASD *vs* HC Sib Lactobacillaceae ↑ (p=0.018), Bifidobacteriaceae ↑ (p=0.0054), and Veillonellaceae ↑ (p=0.008) in ASD *vs* HC Sib Erysipelotrichaceae ↑ (p=0.0005), Enterococcaceae ↑ (p=0.0127), and Desulfovibrionaceae ↑ (p=0.03) in ASD *vs* HC Sib *Bifidobacterium* ↑ (p=0.005), *Lactobacillus* ↑ (p=0.018),	No metabolites were studied	It is not indicated whether the ASD have intellectual disability

				Megasphaera ↑ (p=0.0008) and *Mirsuokella* ↑ (p=0.007) in ASD *vs* HC Sib 99% of *Lactobacillus* was *Lactobacillus ruminis* in ASD group		
Rose et al. (2018)	ASD w FGID (blood: n=20, 15 males, 5 females) (stool: n=21, 17 males, 4 females) ASD w/o FGID (blood: n=26, 19 males, 7 females) (stool: n=29, 25 males, 4 females) Age 3-12 y	HC w FGID (blood: n=6, 5 males, 1 female) (stool: n=7, 6 males, 1 female) HC w/o FGID (blood: n=35, 24 males, 11 females) (stool: n=34, 32 males, 2 females) Age 3-12 y	Sequencing of the 16S rRNA gene bacterial (V3-V4 regions) on fecal samples Multiplexing bead immunoassay for L-1alpha, IL-1beta, IL-6, IL-12 (p40 & p70), TNFalpha, IFNc (TH1), IL-4, IL-13 (TH2), IL-10, TGFbeta1, IL-5, IL-15 and IL-17 cytokines in PBMC from blood samples Immunoblot for Haptoglobin (Hp) genotype	*Bacteroidaceae* ↑, *Lachnospiraceae* ↑, *Ruminococcaceae* ↑ and *Prevotellaceae* ↑ in ASD w GI *vs* HC w GI Il-5, IL-15 and IL-17 ↑ in ASD w FGID *vs* ASD w/o FGID (after exposure to the TLR-4 agonist LPS) TGFbeta1↓ in ASD w FGID *vs* ASD w/o FGID and HC w/o FGID (p<0.05) (under the majority of conditions examined)	No correlation between cytokines levels or Hp and fecal microbiota Differences in the microbiome composition of children with ASD *vs* HC groups, irrespective of FGID	ASD severity is not indicated. It is not indicated whether the ASD have intellectual disability Limited sample size and younger age of the HC with FGID group
Coretti et al. (2018)	ASD (n=11, 9 males, 2 females) Age 2-4 y	HC (n=14, 8 males, 6 females) Age 2-4 y	Sequencing of the 16S rRNA gene bacterial (V3-V4 regions) on fecal samples Droplet Digital PCR (ddPCR)	*Bacteroidetes/Firmicutes ratio* ↑ (p<0.05) in ASD *vs* HC *Actinobacteria* ↓ (p=0.004), *Bacteroidetes* ↑ (p=0.04), *Proteobacteria* ↑ (p=0.004) in ASD *vs* HC *Streptococcaceae, Gemellaceae, Coriobacteriaceae, Bifidobacteriaceae and Actinomycetaceae* ↓ (p=0.004) in ASD *vs* HC *Actinomyces* ↓, *Corynebacterium* ↓, *Bifidobacterium* ↓, *Eggerthella* ↓, *Parabacteroides* ↑, *Streptococcus* ↓, *Ruminococcus* ↑, *Blautia* ↓, *Coprococcus* ↓, *Lachnospira* ↑, *Roseburia* ↑, *Oscillospira* ↑,	*Faecalibacterium prausnitzii, B. uniformis*, were positively correlated to the ADOS score (r=0.767 and r= 0.84, p<0.001) ASD *vs* HC *Faecalibacterium prausnitzii, Ruminococcus torques* and *Eubacterium eligens*, were positively associated to butyrate level Butyrate ↑ (p=0.005) ASD *vs* HC Phosphate butyryltransferase gene ↑, acetate CoA-transferase alpha subunit gene ↑ (p<0.05) ASD *vs* HC *Ruminococcus torques* ↑,	ASD severity is not indicated. It is not indicated whether the ASD have intellectual disability A small number of studied patients and strains

				Faecalibacterium prausnitzii ↑, (p<0.05) in ASD *vs* HC	Beta-hexosaminidase (K12373) (>0.01%) ↑ ASD *vs* HC	
Zhang et al. (2018)	ASD w FGID (n=35, 29 males, 6 females) Age 3-8 y	HC (n=6, 5 males, 1 females) Age 3-8 y	Sequencing of the 16S rRNA gene bacterial (V3-V4 regions) on fecal samples.	*Firmicutes/Bacteroidetes ratio*↑ in ASD w FGID *vs* HC (p<0.05) *Bacteroidetes* ↑ in ASD w FGID *vs* HC (p<0.05) *Firmicutes* ↓ in ASD w FGID *vs* HC (p<0.05) *Veillonella*, *Streptococcus*, *Escherichia*, *Actinomyces*, *Parvimonas*, *Bulleida* and *Peptoniphilus* ↓ in ASD w FGID *vs* HC (p<0.05)	Positive microbe-based link between periodontitis and ASD Negative microbe-based link between type 1 diabetes, constipation, IBS, psoriasis and ASD	ASD severity is not indicated. It is not indicated whether the ASD have intellectual disability A small number of studied patients and strains
Berding and Donovan (2018)	ASD (n=26, 19 males, 7 females) Age 2-7 y	HC (n=32, 19 males, 13 females) Age 2-7 y	Sequencing of the 16S rRNA gene bacterial (V3-V4 regions) on fecal samples. Real time qPCR for total bacteria, *Lactobacillus*, *Bifidobacterium*, *Prevotella*, *Clostridium perfringens*, and *C. difficile* Gas-chromatography mass spectrometry of VFA on fecal samples	Firmicutes ↑ in ASD *vs* HC (p<0.03) Coriobacteriaceae ↑ (p=0.04), Peptostreptococcaceae ↑ (p=0.05) and Rikenellaceae ↓ (p=0.005) in ASD *vs* HC *Clostridium*, *Blautia*, and *Roseburia* ↑ in ASD *vs* HC (p≤0.05) *Butyricimonas*, *Butyrivibrio*, *Faecalibacterium*, *Dialister*, and *Bilophila* ↓ in ASD *vs* HC (p≤0.05) *Bifidobacterium* ↓ in ASD *vs* HC (p=0.04) *C. perfringens* ↓ in ASD *vs* HC (p=0.009)	Intake of insoluble dietary fiber was negatively correlated (r=−0.4; p=0.04) with abundance of Clostridiales. *Faecalibacterium* abundance was positively correlated with servings per day of fried food (r=0.43; p=0.0), but negatively correlated with servings per day of fruit (r=−0.39; p=0.05)	ASD severity is not indicated. It is not indicated whether the ASD have intellectual disability

Note: w = with; w/o = without; ASD = Autism Spectrum Disorder; HC = Healthy control; FGID = Functional Gastro-Intestinal Disorders; NT = Neurotypical; NT Sib = Neurotypical siblings; Sib = siblings; y = years; ADI = Autism Diagnostic Interview; DHEA-S = dehydroepiandrosteronesulfate; PDD-NOS = Pervasive Developmental Disorder Not

Otherwise Specified; IBS = Irritable Bowel Syndrome; BMI = Body Mass Index, FAA = Free Amino Acids, NT=HC, VFA: volatile fatty acids